

# Targeting Toll-Like Receptor 4: a promising strategy to prevent type 1 diabetes occurrence or recurrence

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## ABSTRACT

**TLR4 is a transmembrane receptor of the innate immune system that recognize LPS of gram-negative bacteria. Its stimulation induces pro-inflammatory responses and also modulates adaptive immunity. In this article, we discuss the role of TLR4 in the activation and proliferation of T cells at the onset of autoimmune diabetes. We review the pathways involved in these observations. Finally, we show how targeting TLR4, by a variety of strategies, can prevent the occurrence of the disease but also its recurrence after allogeneic islet of Langerhans transplantation.**

## INTRODUCTION

Type 1 diabetes (T1D) is an autoimmune disease of unknown origin. It represents 5- 10% of the cases of diabetes diagnosed worldwide and mainly affects pediatric patients<sup>1</sup>. It is characterized by autoimmune destruction - of the insulin-secreting cells ( $\beta$ -cells) of the islets of Langerhans. The decreased number of  $\beta$ -cells leads to an insufficient secretion of insulin and consequently to the dysregulation of glucose uptake and metabolism. There are genetic predispositions and environmental factors involved in the onset of the process leading to the destruction of the  $\beta$ -cells<sup>2</sup>. In fact, there is growing evidence to suggest that T1D has remarkable interindividual heterogeneity in terms of clinical and immunopathological features<sup>3</sup>.

In this context, identifying modifiable factors is essential for the set-up of preventive strategies for patients at risk for T1D, but also to avoid recurrence of autoimmunity and allograft rejection after pancreas or islet transplantation and possibly, in the future, after autologous stem cell-derived islet transplantation or other innovative  $\beta$ -cell replacement or regeneration therapies. In this review, we will discuss the importance of chronic inflammation and insulinitis in the pathogenesis of T1D. We will focus on the involvement of Toll-Like Receptor 4 (TLR4), a component of the inflammatory response, in the pathogenesis of T1D and on how it can be targeted for the prevention of the disease occurrence, or recurrence after  $\beta$ -cell replacement strategies.

## TOLL-LIKE RECEPTORS AND AUTOIMMUNE DIABETES

The Toll-Like Receptor (TLR) family consists of 10 members in the human and 12 in the mouse<sup>4</sup>. They are either transmembrane molecules or expressed in intracellular compartments. Each transmembrane TLR is made of leucine-rich repeats (LRRs) at their N-terminal ends and a cytoplasmic Toll/IL-1 receptor (TIR) domain. TLRs are expressed in innate and adaptive immune cells, but also in fibroblasts, epithelial cells and adipocytes. Recently they have been identified in the  $\beta$ -cells<sup>5,6</sup>. As pattern recognition receptors (PRRs), expressed by antigen-presenting cells (APC), they are able to recognize exogenous pathogen-associated molecular patterns (PAMPs), that include bacteria-, virus-, fungus- and parasite- derived components, but are

also able to recognise endogenous damage-associated molecular patterns (DAMPs), such as self-DNA from necrotic or apoptotic cells. TLRs are essential to immune responses against pathogens. However inappropriate activation may promote inflammation and autoimmunity<sup>7</sup>. Recent discoveries have linked TLRs to inflammatory and autoimmune pathologies such as multiple sclerosis, autoimmune uveitis, arthritis, psoriasis, vasculitis, lupus erythematosus, inflammatory bowel disease and autoimmune diabetes<sup>8-12</sup>.

The study of TLRs in autoimmune diabetes is complex. More than one cell, involved in the pathogenesis of T1D, express TLRs in addition to the multiple ligands that can interact with them. Thus, numerous contradictory results on the course of T1D have been published<sup>12</sup>. TLR4 is a member of the TLR family. It complexes with myeloid differentiation factor 2 (MD-2) and recognizes bacterial lipopolysaccharide (LPS). Structural analysis revealed that five of six lipid chains of LPS bind to a hydrophobic pocket in MD-2, while the remaining chain binds to TLR4. TLR4-MD2-LPS complexes dimerize and induce signal transduction by recruiting adaptor proteins containing a TIR domain such as MyD88, TIRAP, TRAM and TRIF<sup>13</sup>. MyD88/TIRAP-dependent pathway induces the production of pro-inflammatory cytokines whereas TRIF/TRAM-dependent pathway is necessary for the production of type I IFN, a large subgroup of proteins that regulate the innate immune system to elicit responses against pathogens or tumors<sup>14</sup>. Although pathogen-recognition receptors in autoimmune diabetes have been widely studied, little is known about the role of TLR4 in the pathogenesis of this disease<sup>12</sup>.

The expression of TLR4 in the islets of Langerhans has been documented to be deleterious both in murine and human  $\beta$ -cells<sup>6,15</sup>. Exposure to LPS induces a loss of  $\beta$ -cells and decreases the synthesis and secretion of insulin<sup>5,6,10,15</sup>. Moreover, it has been observed that the sera of type 1 diabetic patients exhibit increased levels of endogenous TLR4 ligands, such as endotoxins, HSP60 and HMGB1<sup>16</sup>. TLR4 expression is also upregulated on monocytes from type 1 diabetic patients<sup>17</sup>. Additionally, in a human cell model, CXCL10, through TLR4 signaling, induces  $\beta$ -cell death and dysfunction (18). Taken together these data provide convincing evidence for the deleterious effects on  $\beta$ -cells mediated by TLR4 activation.

## TLR4 IN THE NON-OBESE DIABETIC (NOD) MOUSE MODEL

Little is known about the role of TLR4 in the development of autoimmune diabetes and contradictory observations are frequent. In an attempt to address this issue, a TLR4-deficient NOD mouse model has been developed<sup>19</sup>. Glden et al<sup>12</sup> reported that TLR4-deficient NOD mice present an accelerated form of autoimmune diabetes<sup>12</sup>. The mean age of diabetes incidence was 17 weeks in TLR4-deficient NOD mice and 25 weeks in the control group. On the other hand, Devaraj et al<sup>19</sup> described a protective phenotype in another TLR4 knock-out mouse model. Along a similar line, MyD88-deficient NOD mice, with impaired TLR4 signaling, do not develop diabetes<sup>20</sup>. Kim et al<sup>7</sup> also reported that the median age of diabetes onset was not affected by TLR4 gene knock-out. Moreover, the prevalence of diabetes was similar between TLR4<sup>+/+</sup>, TLR4<sup>+/-</sup> and TLR4<sup>-/-</sup> mice<sup>12</sup>.

The TLR4-deficient NOD mouse model is a useful tool to study the involvement of TLR4 in the pathogenesis of autoimmune diabetes. However, a general knockout of TLR4 appears to be not sufficient to understand the complex crosstalk between TLR4-expressing cells and to define their putative role in the onset of autoimmunity.

So far, the cell-specific role of TLR4 engagement has mainly been studied in  $\beta$ -cells and APC. The activation of TLR4 in murine  $\beta$ -cells impairs the insulin secretion and increases  $\beta$ -cell apoptosis in a murine model of type 2 diabetes<sup>21</sup>. On the other hand, TLR4 blockade preserves the survival of human and murine islets in the presence of LPS<sup>15</sup>. In this context, the engagement of TLR4 in  $\beta$ -cells may have a direct damaging effect.

TLR4 is mainly expressed in APC and its activation increases phagocytosis, antigen presentation and secretion of pro-inflammatory cytokines<sup>22</sup>. Macrophages can be found at the early stages of islet infiltration and probably play an important role in the onset of autoimmune diabetes<sup>23,24</sup>. The activation of TLR4 in APC increases the secretion of pro-inflammatory cytokines in diabetic mice<sup>21</sup>. On the contrary, TLR4-deficient macrophages show reduced pro-inflammatory cytokine secretion and unresponsiveness to LPS exposition in NOD mice<sup>12</sup>. On the basis of this evidence TLR4-deficient APC in NOD mice could have a possible protective phenotype against autoreactivity.

## PREVENTION OF AUTOIMMUNE DIABETES BY TLR4 INHIBITION

Because of these considerations, we have explored whether TLR4 blockade could prevent the development of insulinitis and the onset of autoimmune diabetes in NOD mice (25). Using CLI-095, a cyclohexene derivative that inhibits TLR4 signaling, we were able to observe a protective effect mediated by the decreased activation and proliferation of the diabetogenic autoreactive CD4<sup>+</sup> T lymphocytes as well as IFN- $\gamma$  production by these cells, both in the pancreatic lymph nodes and in the spleen, and consequently the inhibition of the autoimmune process. Interestingly, this was specifically observed in CD4, but not CD8 T-cells<sup>25</sup>.

We further investigated the impact of TLR4 inhibition on CD4<sup>+</sup> T lymphocytes in an adoptive transfer model of autoimmune diabetes. In this model, naïve NOD/BDC2.5 CD4<sup>+</sup> T lymphocytes, expressing a rearranged TCR $\alpha\beta$ , are transferred to immunocompromised NOD/rag1. CLI-095 treatment decreased the activation of CD4<sup>+</sup> T lymphocytes and the number of IFN- $\gamma$ - and IL-17A-producing CD4<sup>+</sup> T lymphocytes, as compared to control. As observed in the NOD mouse model, TLR4 blockade impaired CD4<sup>+</sup> T lymphocytes activation, proliferation and differentiation into diabetogenic effector cells, both in the pancreatic lymph nodes and in the spleen<sup>25</sup>.

Since the injections of the CLI-095 TLR4 inhibitor were systemically (intraperitoneally) administered, numerous cells could have been targeted. This protective effect is potentially not only due to its effect on CD4<sup>+</sup> T lymphocytes, but also on APCs. A protective effect on  $\beta$ -cells is also possible. Therefore, TLR4 inhibition could have the dual advantage of targeting the immune cells involved in the pathogenesis of T1D and directly increasing the viability of  $\beta$ -cells. Moreover, TLR4 is the receptor for inflammatory mediators (HMGB1, DAMPs, CXCL10), autoantigens (Heat-shock protein 60; HSP60) but also bacterial LPS, all being involved in the pathogenesis of T1D. Therefore, targeting inflammatory and autoimmune processes involved in the pathogenesis of T1D, using one single treatment could synergize the therapeutic efficiency and explain the promising results observed in NOD mice.

CLI-095 (also known as TAK-242) and Eritoran, are 2 inhibitors of TLR4 signaling, which have already been used in phase 3 clinical trials,

in patients with severe sepsis<sup>26,27</sup>. Regardless of the negative results of the trials, these TLR4 inhibitors were well tolerated. Therefore, inhibiting TLR4 by either of these agents in patients at risk for T1D could be a valid and feasible intervention. A derivative of HSP60 targeting TLR2 already demonstrated promising results on the preservation of  $\beta$ -cell function in newly diagnosed T1D patients<sup>28</sup>. In addition, TLR polymorphisms are associated with susceptibility to infectious, inflammatory and autoimmune diseases<sup>29</sup>. Because of the highly-conserved TLR functions throughout animal species, findings on the roles of TLRs in murine models could be easily translated to humans. Numerous clinical trials targeting TLR2,3,4,5,7,8 and TLR9 are ongoing for prevention and treatment of various cancers, allergic diseases, as well as inflammatory and autoimmune diseases<sup>30</sup>.

## PREVENTION OF DIABETES RECURRENCE BY TLR4 BLOCKADE AFTER ISLET TRANSPLANTATION

TLRs provide a well-recognized link between the innate and adaptive immune systems. In transplantation, they are a key link between ischemia-reperfusion injury and graft rejection<sup>31-33</sup>. They play this role by enhancing antigen presentation, upregulating costimulatory molecule expression and stimulating proinflammatory cytokine and chemokine production. TLRs are typically engaged by exogenous ligands (such as LPS) in infection, but are also able to sense endogenous ligands, collectively known as damage-associated molecular patterns (DAMPs), such as key molecules involved in autoimmune diseases<sup>34,35</sup> or necrotic cell products, usually released during ischemia-reperfusion injury in organ or cell transplantation<sup>31,34</sup>.

TLRs were shown to play an important role in the inflammatory phenomena observed in the first stages of intraportal islet transplantation and leading to the early destruction of significant number of implanting endocrine cells. In particular, islet cells constitutively express TLRs, notably TLR2 and TLR4, and the isolation process upregulates TLR4 expression at their surface<sup>36,37</sup>. Further, the engagement of islet-expressed TLRs by DAMPs at the time of transplantation leads to a massive release of proinflammatory cytokines and chemokines that contribute to the immediate destruction of a sizeable amount of implanting islet cells<sup>36,38</sup>. The role of TLRs in these

processes was evidenced by experiments of syngeneic islet transplantation in TLR4<sup>-/-</sup> murine models. Interestingly, absence of expression of TLR4 in the islet donors, not the recipients led to better engraftment, indicating that TLR4 expression by the islets, not the host innate immune system, was responsible for this phenomenon, which was largely mediated by the inhibition of NF-kappaB activation<sup>36,38</sup>.

From another standpoint, TLR4-mediated dendritic cell (DC) activation can lead, via the nuclear factor kappa B pathway, to DC maturation and Treg suppression<sup>39</sup>. In the setting of transplantation, ischemic injury can activate donor DCs by means of heat-shock protein70-TLR4 interaction. Donor-derived activated DCs can enhance immunogenicity of the graft, thus promoting graft rejection<sup>31,40</sup>. Therefore, in addition to its impact on controlling autoimmune diabetes, TLR4 blockade could have the potential to mitigate the effects of islet or pancreas graft allogeneic immunogenicity.

There have been contradictory reports about the effect of TLR4 in allogeneic islet graft rejection, utilizing TLR4<sup>-/-</sup> murine models. While all publications concur in finding a beneficial role of TLR4 inactivation on allogeneic islet graft rejection, significant differences are observed, depending on experimental design. Reports show indefinite islet graft survival in TLR4<sup>-/-</sup> to wild type transplants, while others show the opposite (37,40,41). The different mouse strain combinations used may partly explain these discrepancies. They also exemplify the limitations of genetic manipulation and the differences between constitutional lack of expression and active blockade.

With an intent to develop an interventional strategy, we have explored the effect of a pharmacological TLR4 blockade, using monoclonal antibodies<sup>15</sup>. In mixed lymphocyte-islet reaction (MLIR) experiments, in which islets were co-cultured with allogeneic mononuclear cells, we were able to inhibit T-cell proliferation and IFN- $\gamma$  expression with an anti-TLR4 monoclonal antibody (mAb). Interestingly, identical results were achieved in these *in vitro* experiments, using murine or human materials and a species-specific mAb<sup>15</sup>. *In vivo*, TLR4 blockade allowed indefinite allogeneic islet graft survival in a majority of animals. This effect was achieved only when both islet donors and islet graft recipients were treated with the mAb, indicated that TLR4 had to be inhibited both in the transplanted islet cells and the host immune system in order to

achieve maximal effect<sup>15</sup>. In further mechanistic studies, animals tolerant to the islet graft (>100 days) were re-challenged with donor-specific or third party skin grafts. Donor-specific skin transplantation led to skin and islet graft loss within 12 days, whereas only skin got rejected, in the same time frame, after third party skin transplantation. This indicated that the protective effect of TLR4 blockade led to a phenomenon of graft accommodation rather than true immune tolerance<sup>15</sup>.

It should be noted here that, in these murine experiments, islets were transplanted under the kidney capsule, unlike the current practice in clinical islet transplantation, where islets are injected into the liver via the portal vein. A significant portion of transplanted islets are thought to be destroyed very early after injection by a phenomenon known as IBMIR ("Instant Blood-Mediated Inflammatory Reaction"), in which a cascade of inflammatory events is elicited by intravascular coagulation within the portal vein<sup>42</sup>. Involvement of TLRs in this phenomenon was reported, which makes it very likely that TLR4 blockade could enhance islet engraftment by controlling, or even inhibiting IBMIR<sup>43</sup>.

#### TOLL-LIKE RECEPTOR BLOCKADE: TOWARD CLINICAL APPLICATION?

The literature reviewed in this article shows a clear potential for TLR -and in particular TLR4- blocking agents, both for the prevention of type 1 diabetes and also as a strategy aiming at promoting and prolonging islet graft survival. The mechanisms of action equally involve the targeting of inflammatory phenomena mediated by the innate immune system, but also purely adaptive immunity, such as occurs in autoimmunity and allogeneic graft rejection.

It is of interest that TLR4 blockade can be achieved in humans by a variety of agents, such as CLI-095 and Eritoran, both already tested in clinical trials<sup>26,27</sup>, but also by human-specific monoclonal antibodies, which have been tested for another type of autoimmune disease (rheumatoid arthritis) in phase II studies<sup>44</sup>. The availability of these compounds makes the clinical application of TLR4 targeting an immediate possibility for clinical trials aimed to prevent type 1 diabetes occurrence in subjects at risk and to enhance islet graft implantation and survival after pancreatic islet allotransplantation.

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The authors have no conflict of interest to disclose.

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